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<p>The gene for ribulose-bisphosphate carboxylase was characterized for several chemoautotrophic bacterial symbionts. The gene of the symbiont of the deep-sea snail <u>Alvinoschisma hessleri</u> has been largely sequenced and compared to the sequences of other photo- and chemoautotrophic bacteria and preliminary phylogenetic tree has been constructed.</p> <p>Oligonucleotide probes against the symbionts in <u>Lyrodus pedicellatus</u> have been used to verify the identity of this bacterium.</p> <p>Method development to obtain sequences from badly preserved symbiont/host samples has been continued. See p. 18 for work done.</p>					
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ANNUAL PROGRESS REPORT ON CONTRACT N00014-88-K-0079; R&T 4412026

PRINCIPAL INVESTIGATOR: Horst Felbeck

CONTRACTOR: University of California

CONTRACT TITLE: Biology of Symbioses Between Marine Invertebrates and Intracellular Bacteria

START DATE: 1 January 1988

**RESEARCH OBJECTIVE:** To study the properties of symbiotic bacteria and to characterize them by 16S rRNA sequencing

**PROGRESS:**

Our research focused mainly on three subjects during this report period:

- a) The characterization and sequencing of the gene for ribulose biphosphate carboxylase from symbiotic bacteria of various origins,
- b) The application of labeled oligonucleotide probes to localize specific symbiotic bacteria in host tissues, and
- c) To continue methods development for 16S rRNA sequencing from symbionts in frozen and badly preserved specimens.

**Ribulose-1,5,-biphosphate carboxylase**

We designed from published sequence information oligonucleotide primers which are complementary to conserved regions on RubisCO large and small subunit genes. These primers were used successfully to amplify using polymerase chain reaction (PCR) specific regions of RubisCO including both subunit genes from genomic DNA samples containing a mixture of host and symbiont DNA from:

- the Mariana Gastropod *Alvinochonca hessleri*
- the gutless clam *Solemya reidi*
- the hydrothermal vent clam *Calyptogena magnifica*
- the sulfur oxidizing bacterium *Thiomicrospira crunegena* isolated from hydrothermal vents and the species most closely related (based on 16S rRNA sequences) to the symbionts

The hybridization of heterologous gene probes for the large subunit of ribulose biphosphate carboxylase/oxygenase (RubisCO) to symbiont DNA shows that the symbionts from both deep-sea and shallow-water invertebrates possess RubisCO genes with a high degree of homology with either a cyanobacterial (*Anabaena*) or a photosynthetic bacterial (*Rhodospillum rubrum*) RubisCO probe. Two deep-sea hydrothermal vent invertebrates, the vestimentiferan tubeworm *Riftia pachyptila* and the gastropod mollusc *Alvinochonca hessleri*, contain symbionts with RubisCO genes homologous to both the cyanobacterial and the photosynthetic bacterial probes

suggesting that these invertebrates possess either two symbionts each with a different RubisCO gene or a single symbiont containing two RubisCO genes. The cyanobacterial-type symbiont RubisCO genes were further distinguished via PCR amplification of the large and small subunit genes. Amplified large subunit gene regions display a strong conservation in size amongst the symbionts and *Anabaena*. By contrast, variation in size of the large plus small subunit gene region amplified from the symbionts and *Anabaena* suggests that the symbionts possess a smaller intergenic region than the cyanobacterium.

The cyanobacterial-type RubisCO gene of the *Alvinoschisma hessleri* symbiont was selected for more detailed study by DNA sequence analysis. Because this organism occurs at a depth of 4,000 meters we suspect that its RubisCO enzyme may possess adaptations to pressure which would be reflected in the enzyme's gene sequence. Total DNA extracted from the symbiont-containing gill tissue was used to construct a genomic library in lambda zap. This library was screened for RubisCO recombinant clones with a large subunit probe from *Anabaena* 7120. Three positive plaques were selected for in-vivo excision into pBluescript phagemid. One of these, designated pAH53, contained an 8.5 kb EcoRI fragment which hybridized strongly to the *Anabaena* probe. Restriction mapping of this fragment showed that the RubisCO large subunit gene resided on a 4.5 kb EcoRI/BamHI fragment. This fragment was subcloned into pBluescript II KS+ and the resultant recombinant clone was designated pAH4.5. Synthetic oligonucleotide primers directed against the highly conserved regions in RubisCO, representing the active and subunit binding sites, were used for sequencing primers in addition to the T3 and T7 primers which were used to initiate sequence from pBluescript regions flanking the insert. Double stranded sequencing reactions using Sequenase were performed and the resultant sequences were compared with those stored in Genbank.

Analysis of this sequence shows that both large and small subunits of RubisCO are present in pAH4.5. A phylogenetic comparison of large subunit sequence obtained to date suggests that the *A. hessleri* symbiont RubisCO is more closely affiliated with that of photoautotroph than that of another chemoautotroph *Alcaligenes eutrophus*.

### Oligonucleotide probes

We have completed a project to identify and localize symbiotic bacteria in host tissue using fluorescently labeled oligonucleotide probes. This project had been started by Dr. Distel two years ago and uses probes against the bacterial symbionts of shipboreworms. These symbionts are an excellent model for the chemoautotrophic symbionts because they also live intracellularly. Contrary to the chemoautotrophs, however, they have been cultured successfully and have been characterized systematically. Using these probes he could show that the cultured bacteria are indeed the genuine symbionts of the shipboreworm *Lyrodus pedicellatus*.

### Sequencing

We had developed a way to extract and purify the gene for the 16S rRNA from badly preserved tissues. Since 16S rRNA itself was usually badly preserved in these samples we expected this a promising way to gather sequence information from the bacterial symbionts. The approach used was to amplify the respective genes by PCR



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and then sequence the amplification products. However, controls showed that sometimes contaminating DNA was more strongly amplified than the symbiont nucleic acids. Because of the numerous controls necessary to establish the identity of the amplified DNA several PCR clones had to be simultaneously sequenced to verify the identity of the sequences. This approach turned out to be very time consuming and did not result in a fast method to obtain 16S rRNA sequences. We, therefore, started a different method based on directed cDNA cloning in which ss rRNA specific primers are used to create a directed cDNA library of the ss rRNA present in the sample followed by cloning and sequencing of these cDNAs. The advantage of this technique is that the cDNA libraries reflect the copy number of the ss rRNAs in the sample thus voiding the problem of changing the quantitative ratios of rRNA in the mixed samples.

### **WORK PLAN (YEAR 3):**

We will have the complete RubisCO sequence for the A. hessleri symbiont within the next few weeks. From this sequence additional PCR primers will be constructed in order to amplify and sequence portions of RubisCO genes from a variety of symbionts with the aim of determining the phylogenetic relationship of symbionts from different hosts and locations. The gene for the nitrogen fixing enzyme nitrogenase will also be characterized and at least partially sequenced from our library of purified large molecular weight DNA of a wide variety of symbionts pending the outcome of the PCR amplification trials using newly constructed primers.

Methods development will proceed to sequence symbiont 16S rRNAs from various symbiotic systems and more sequences will be obtained.

### **PUBLICATIONS AND REPORTS (YEAR 2)**

Cary, S.C., Novitsky, T.J., Hentschel, U., Felbeck, H.: Biomass assessment of bacterial endosymbionts in specialized tissues of marine invertebrates using the Limulus lysate assay to monitor lipopolysaccharides. Mar. Biol., in press

Felbeck, H., and D. L. Distel: Prokaryotic symbionts in marine invertebrates. In: The Prokaryotes, 2nd edition (eds. A. Balows, H.G. Trüper, M. Dworkin, W. Harder, K. H. Schleifer), Springer Verl., in press

Felbeck, H.: Symbiosis of bacteria with invertebrates in the deep sea. Endocytobiology IV, Symposium volume, 8 pages, invited talk and in press

Stein, J., Haygood, M., and H. Felbeck: Diversity of ribulose 1,5 bisphosphate carboxylase in thiotrophic symbioses. Endocytobiology IV, Symposium volume, 4 pages, in press

Distel, D. L.: Detection, Identification and phylogenetic analysis of endosymbiotic bacteria using ribosomal RNA sequences. Endocytobiology IV, Symposium volume, 4 pages, in press

### **TRAINING ACTIVITIES:**

Research assistantship for Ute Hentschel (50%, part of the year) and salary for postdoctoral researcher Dr. Daniel L. Distel.

**AWARDS**

Graduate student Jeffrey Stein has been awarded a NASA graduate student fellowship. He is still working on the research described in the ONR contract

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